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TECHNICAL MANUSCRIPT 265

IMMUNOLOGICAL OVERLAP AND
IDENTIFICATION OF GROUP A ARBOVIRUSES
BY A GRADIENT PLATE PLAQUE TECHNIQUE

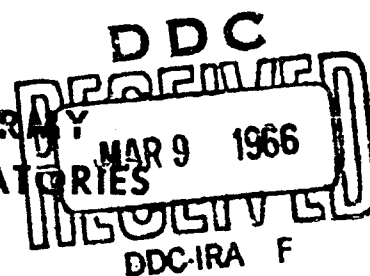
Julius E. Officer, Jr.

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Arthur Brown

JANUARY 1966

UNITED STATES ARMY
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IMMUNOLOGICAL OVERLAP AND IDENTIFICATION OF GROUP A ARBOVIRUSES
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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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ABSTRACT

A semi-quantitative, gradient plate, plaque-neutralization technique was developed for studying immunological overlap and identification of Group A arboviruses. Two principles of the technique are: (i) a concentration gradient of antibody is provided that continues into a control area without antibody, and (ii) neutralization is measured by inhibition of plaque formation in agar-cell suspension. The virus sample is streaked over an agar surface with an inoculating loop so that the line of plaques extends from control area into antibody-gradient area to provide an estimate of plaque neutralization overlap. Venezuelan (VEE), eastern (EEE), western (WEE) equine encephalitis and Sindbis viruses were studied. Results showed that reciprocal cross-neutralization occurred between EEE, WEE, and Sinbis virus with one exception of a one-way cross. VEE did not cross with any virus except possibly EEE using anti-VEE serum. In a comparison with two quantitative plaque-neutralization methods, the gradient method gave qualitative results similar to the quantitative method in which antiserum was incorporated into the agar. However, it showed cross-neutralization among more viruses than the quantitative method, which depends upon incubation of virus and antibody prior to plating for reduction of plaques.

I. INTRODUCTION

A number of in vivo and in vitro techniques have been utilized to identify arboviruses and to show immunological relationships within this major serological group and sub-groups. One popular and useful tissue culture technique is based on the neutralization of arboviruses by the simple, rapid, and economical Porterfield plaque-inhibition test. The gradient plate¹ plaque-neutralization technique described in this paper is offered as an alternative to Porterfield's² plaque-inhibition test.

The technique involves two main principles: (i) the inhibition of virus plaque formation by the presence of antibody, similar to Porterfield's except that a cell suspension is used rather than a monolayer; and (ii) a concentration gradient of antibody that is continuous with a control area without antibody. The technique permits streaking a virus sample over an agar surface with a bacteriological inoculating loop so that the length of the line of plaques extends from the control area into the antibody-containing area and provides a semiquantitative estimate of plaque neutralization and immunological overlap.

II. MATERIALS AND METHODS

A. VIRUSES

Members of Group A arboviruses used were western equine encephalitis (WEE), eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE), and Sindbis viruses. They were selected on the basis of experience or data in the literature indicating that there would be only specific neutralization with VEE virus, but there would be overlap with WEE and Sindbis viruses. EEE virus was also included in the test, but the extent of its overlap with the others was uncertain.

Antisera to each of the viral strains were prepared in mice, chickens, guinea pigs, rabbits, and monkeys. The suspended cell plaque method we have described previously³ was used as the basis of the present gradient-plate plaque technique. The plastic dish used in these studies contained four compartments. The diagram in Figure 1 shows the composition of a typical compartment.

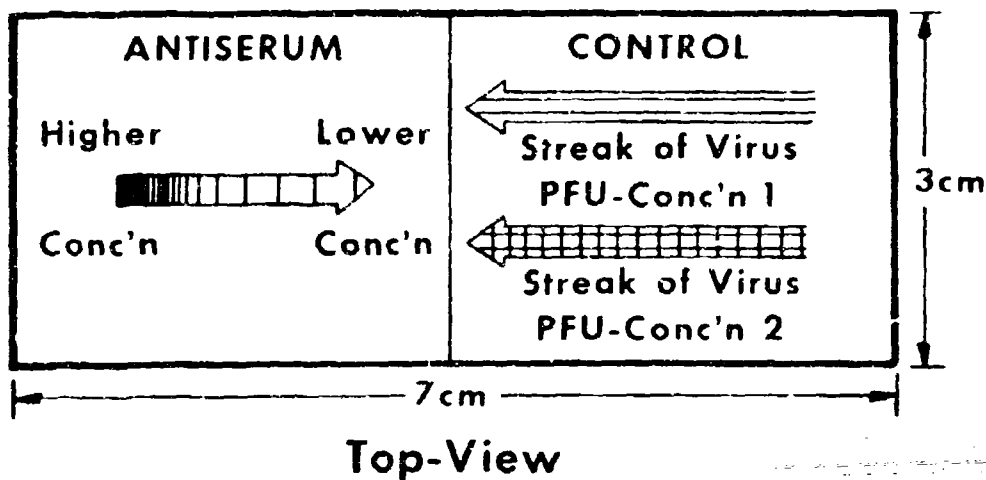
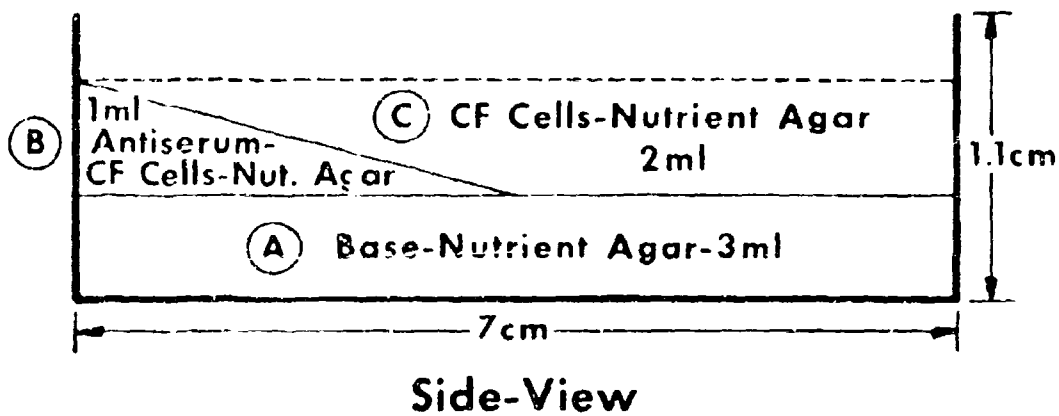


Figure 1. Diagram of Gradient Plate.

Three ml of a lactalbumin hydrolysate-yeast extract-gelatin nutrient agar was added to the compartment as a basal layer. The plate was then tilted and one ml of nutrient agar containing suspended chick fibroblast (CF) cells and antiserum was added to a final concentration of either 1% or 10%. When this layer had solidified, the plate was leveled and 2 ml of nutrient agar containing suspended cells was added. Each compartment of the plate contains one type of antiserum at a given concentration gradient. Two streaks of virus were made on each compartment of the petri dish with a bacteriological loop containing 0.01 ml of suspension. One virus streak usually contained 10^4 plaque-forming units (pfu) and the other 10^6 pfu. A photograph of the plate used in the test is shown in Figure 2.

The plates were stained with a 1:10,000 solution of neutral red in nutrient agar after 24 hours and the results were recorded at 48 hours. When no serum neutralization occurred, the viral streak extended across the entire antiserum gradient and was designated 0 with respect to the degree of neutralization. When the formation of the streak was inhibited at the beginning of the gradient, the virus was completely neutralized and the results recorded as 4+. Intermediate neutralization occurred when the streaks extended partially over the antiserum-containing portion of the gradient. These were graded from 1+ to 3+, depending on their length.

III. RESULTS

The data from several experiments may be seen on the next few tables. Table 1 shows that antisera to WEE completely neutralized the homologous viral strain and also cross-neutralized Sindbis virus, and to a lesser degree EEE virus. VEE virus was not neutralized. The degree of neutralization was found to be dependent both on antiserum concentration and virus concentration.

Anti-Sindbis serum effectively neutralized its homologous strains, as shown in Table 2. The antiserum prepared in chickens partially cross-neutralized EEE at the higher concentrations. None of the other antisera showed any cross-neutralization. The degree of cross-neutralization may be a matter of antibody titer, the kind of vaccine used, the number of vaccine injections, or the host used for immunization.

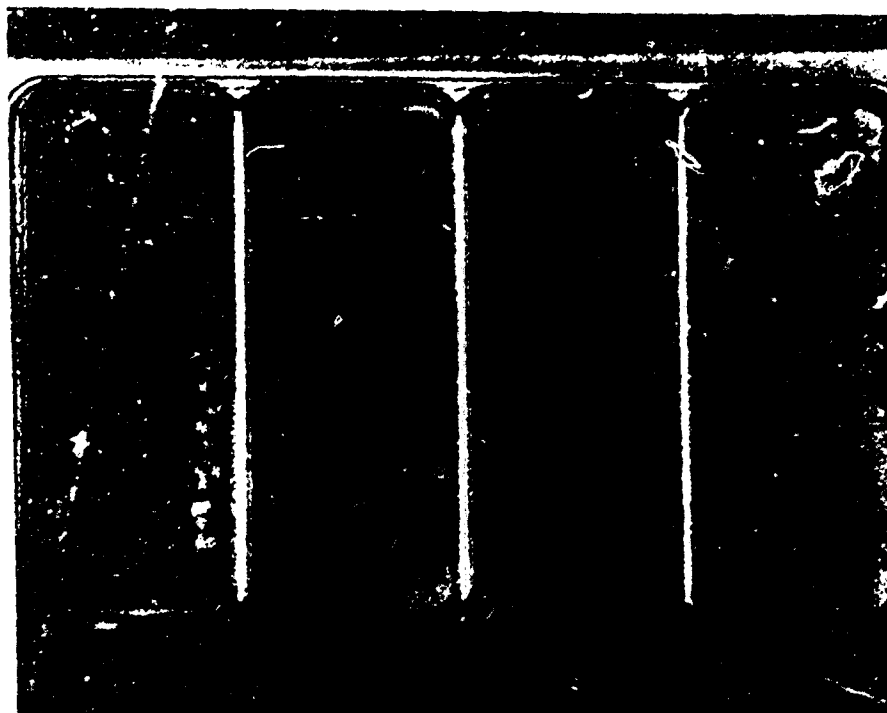


Figure 2. Gradient Plate Containing Anti-Western Equine Encephalitis Virus Serum. Chambers from left to right are streaked vertically with

1. Eastern equine encephalitis virus
2. Sindbis virus
3. Western equine encephalitis virus
4. Venezuelan equine encephalitis virus

TABLE 1. CROSS-NEUTRALIZATION BETWEEN ANTI-WEE SERUM AND OTHER MEMBERS OF GROUP A ARBOVIRUSES

Antiserum Species	Antiserum Dilution	Virus Strain and Concentration (pfu) Streaked ^{a/}							
		WEE		Sindbis		EEE		VEE	
		10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶
Chicken (Inactivated) ^{b/}	10 ⁻¹	4+	4+	4+	2+	4+	4+	0	0
	10 ⁻²	4+	4+	3+	0	0	0	0	0
	10 ⁻¹	4+	-	4+	-	2+	-	0	-
Mouse	10 ⁻¹	4+	4+	4+	3+	4+	0	0	0
	10 ⁻²	4+	4+	4+	1+	2+	0	0	0
Mouse (373)	10 ⁻¹	4+	3+	0	0	1+	0	0	0
	10 ⁻²	3+	1+	0	0	±	0	0	0
Guinea Pig	10 ⁻¹	4+	4+	3+	0	4+	0	0	0
	10 ⁻²	4+	4+	3+	0	1+	0	0	0
Monkey	10 ⁻¹	4+	4+	4+	3+	3+	4+	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0

a. 4+ = complete neutralization. 0 = no neutralization. - = not done.

b. Serum inactivated for 1/2 hour at 56 C.

TABLE 2. CROSS-NEUTRALIZATION BETWEEN ANTI-SINDBIS SERUM AND OTHER MEMBERS OF GROUP A ARBOVIRUSES

Antiserum	Antiserum Dilution	Virus Strain and Concentration (pfu) Streaked ^{a/}							
		Sindbis		WEE		EEE		VEE	
		10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶
Chicken (Inactivated) ^{b/}	10 ⁻¹	4+	4+	1+	0	4+	4+	0	0
	10 ⁻²	4+	3+	0	0	0	0	0	0
	10 ⁻¹	4+	-	2+	-	2+	-	0	-
Mouse 946	10 ⁻¹	4+	4+	0	0	0	0	0	0
	10 ⁻²	4+	3+	0	0	0	0	0	0
Monkey (1243)	10 ⁻¹	4+	0	0	0	0	0	0	0
	10 ⁻²	3+	0	0	0	0	0	0	0

a. 4+ = complete neutralization. 0 = no neutralization. - = not done.

b. Serum inactivated for 1/2 hour at 56 C.

Table 3 shows that anti-EEE serum from a number of sources shows excellent homologous neutralization, and a few cross-neutralized with Sindbis virus.

TABLE 3. CROSS-NEUTRALIZATION BETWEEN ANTI-EEE SERUM AND OTHER MEMBERS OF GROUP A ARBOVIRUSES

Antiserum Species	Antiserum Dilution	Virus Strain and Concentration (pfu) Streaked ^a /							
		EEE		Sindbis		WEE		VEE	
		10 ⁴	10 ⁵	10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶
Chicken (Inactivated) ^b /	10 ⁻¹	4+	4+	1+	1+	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0
	10 ⁻¹	4+	-	2+	-	0	-	0	-
Mouse	10 ⁻¹	4+	3+	0	0	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0
Guinea Pig	10 ⁻¹	4+	4+	0	0	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0
Monkey	10 ⁻¹	4+	4+	0	0	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0
Guinea Pig (1A)	10 ⁻¹	4+	4+	0	0	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0
Rabbit	10 ⁻¹	4+	4+	0	0	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0

a. 4+ = complete neutralization. 0 = no neutralization. - = not done.

b. Serum inactivated for 1/2 hour at 56 C.

Table 4 shows that there is excellent homologous neutralization between anti-VEE serum and VEE virus, and slight cross-neutralization with EEE virus only.

The results obtained with this semi-quantitative, gradient plaque technique were compared with other more quantitative plaque-neutralization techniques. For this purpose, two other techniques were employed in which chicken antisera against the three viruses showing cross-neutralization were selected for comparative tests. The first technique was a suspension plaque-suppression technique in which different dilutions of antiserum were incorporated directly into the agar without any gradient, and the virus was then inoculated onto the hardened agar surface. The antiserum concentration that suppressed 50% of the plaques was calculated and taken as the end point. The second technique was a plaque-reduction type of neutralization technique in which a known number of plaque-forming units of virus was incubated for 30 minutes in a test tube with varying concentrations of antiserum and then diluted and plated on the surface of suspended chick fibroblasts agar plates for reduction of plaques to a 50% end point.

TABLE 4. CROSS-NEUTRALIZATION BETWEEN ANTI-VEE SERUM AND OTHER MEMBERS OF GROUP A ARBOVIRUSES

Antiserum Species	Antiserum Dilution	Virus Strain and Concentration (pfu) Streaked a/							
		VEE		EEE		WEE		Sindbis	
		10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶
Chicken	10 ⁻¹	4+	4+	0	0	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0
Mouse	10 ⁻¹	4+	4+	0	0	0	0	0	0
	10 ⁻²	4+	3+	0	0	0	0	0	0
Monkey	10 ⁻¹	4+	4+	±	0	0	0	0	0
	10 ⁻²	4+	4+	0	0	0	0	0	0
Donkey	10 ⁻¹	4+	4+	1+	0	0	0	0	0
	10 ⁻²	4+	3+	2+	0	0	0	0	0
Human	10 ⁻¹	4+	4+	1+	0	0	0	0	0
	10 ⁻²	3+	1+	0	0	0	0	0	0

a. 4+ = complete neutralization. 0 = no neutralization. - = not done.

Table 5, in comparison with the previous tables, shows that the technique in which the antiserum was incorporated into the agar gave approximately the same qualitative results, i.e., the same cross-reaction as the gradient plate technique, but with the possible addition that the cross-reactions were somewhat more sensitively and quantitatively determined.

TABLE 5. QUANTITATIVE SERUM NEUTRALIZATION TEST
BY ANTISERUM INCORPORATION IN AGAR

Virus Strain	Chicken Antiserum	Log ₁₀ Titer Antiserum (50% Plaque Reduction)
WEE	Anti-WEE	>3.0
Sindbis		2.0
EEE		2.2
WEE	Anti-Sindbis	1.1
Sindbis		>3.0
EEE		2.2
WEE	Anti-EEE	<1.0
Sindbis		1.4
EEE		>3.0

Table 6 shows the results obtained with an in vitro plaque-reduction technique. Here the only cross-reaction was between anti-WEE serum and Sindbis virus. Thus, although this technique is quantitative, it is less sensitive in detecting cross-reactions than either of the former two techniques.

TABLE 6. NEUTRALIZATION OF GROUP A AREOVIRUSES
BY PLAQUE REDUCTION IN VITRO

Virus Strain	Chicken Antiserum	Log ₁₀ Titer Antiserum (50% Plaque Reduction)
WEE	Anti-WEE	4.7
Sindbis		1.5
EEE		<1.0
WEE	Anti-Sindbis	<1.0
Sindbis		3.3
EEE		<1.0
WEE	Anti-EEE	<1.0
Sindbis		<1.0
EEE		3.7

IV. DISCUSSION AND SUMMARY

Our results have shown cross-neutralization reactions between WEE, EEE, and Sindbis viruses; some of these are reciprocal crosses, but others cross-react only one way. Reciprocal crossing between these three viruses and others in Group A may be found in Casals' data⁴ in HI tests using hyperimmune antiserum produced by multiple injection of killed vaccines. Ordinary, single-injection antisera failed to show cross-reactions among most of the group. Porterfield's data² obtained by his simplest, but least sensitive, neutralization methods using beads, show cross-neutralization between Sindbis and WEE with anti-Sindbis serum only, but not all of his antisera were hyperimmune. For the most part, our antisera were hyperimmune.

The gradient-plate, plaque-neutralization method reported here, although semi-quantitative and less economical in the amount of antiserum required, appears to be more sensitive than the plaque-inhibition method used by Porterfield, yet is equally simple to carry out. Unlike cell monolayer techniques, it can be started the same day the cell suspension is prepared. Moreover, the test presented here gives approximately the same qualitative results as more laborious quantitative tests. We are now trying to make our technique completely quantitative.

For diagnostic purposes, the gradient plate, plaque-neutralization method does not require the use of high-titered virus inoculum. The gradient-plate plaque technique appears worthy of further exploration for use with any virus that can make a plaque, especially those that do so within a few days.

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